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The primary objective of this project is to determine the ultrastructural and selected cytochemical effects of diethylnitrosamine (DENA) and trichloroethylene (TCE) on hepatic and renal tissues of the Japanese medaka, Oryzias latipes. Another critical phase of the investigation is to determine whether DENA can function as an inducer of cellular transformation for TCE. One group of 60 medaka was exposed to DENA (10mg/L) for 48hrs, rinsed and maintained in a tank of fresh water. Another group of 60 fish has been continuously exposed to TCE (10mg/L). A third group of medaka was exposed to DENA (10mg/L) for 48hrs, rinsed in fresh water and subsequently has been continuously exposed to TCE (10mg/L). A final group was placed in fresh water and served as a control. The sacrifices have been scheduled at 2,4,6 and 12 months subsequent to the initial exposure to DENA. At this point in the study, the first two sacrifices have been conducted and the tissues appropriately processed for electron microscopy. The specific enzymes selected for analysis by means of electron-dense markers are acid phosphatase, peroxidase and ATPase. Samples collected at the first two sacrifices have been processed. Control liver is the (continued on back)

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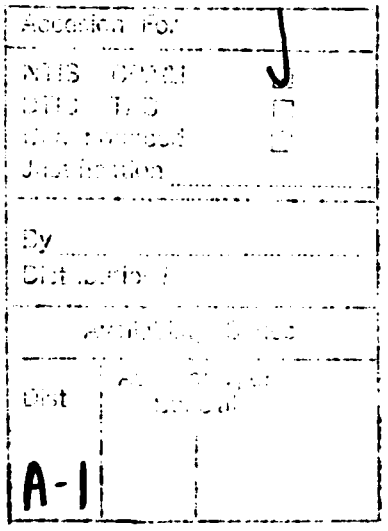
only tissue which has been sectioned and investigated at this point in the study. The ultrastructural features are comparable to those described for the liver of medaka in several scientific articles. The Principal Investigator is currently investigating tissues selected for cytochemical analysis.



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N/A In conducting research using animals, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council (NIH Publication No. 86-23, Revised 1985).

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William D. Norton 2/24/91

PI Signature Date

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INTRODUCTION

Interest in the application of non-mammalian model system in environmental toxicology and carcinogenesis studies is expanding rapidly (Masahito et al., 1988; Hinton et al., 1988). Several species of fish are recognized as acceptable and effective models for toxicology and carcinogenesis investigations and as complementary extensions of current mammalian testing systems (Couch & Courtney, 1987; Hawkins et al., 1986). Fish have exhibited relative degrees of susceptibility to a variety of unrelated carcinogens. The Japanese medaka, Oryzias latipes has served as a popular model for the investigation of chemically induced hepatic and renal tumors (Hinton et al., 1988; Norton & Gardner, 1987). Certain studies have included the use of diethylnitrosamine, a site specific carcinogen for hepatic neoplasms (Kimura et al., 1981; Kyono, 1978).

Since the employment of fish as a bioassay system is a relatively new approach to the investigation of cellular transformation, there are considerable voids in our basic knowledge of the biochemical, cytochemical and ultrastructural manifestations of exposure to selected carcinogens. Specifically, there is a paucity of information regarding cellular alterations which result from fish being exposed to different compounds in a sequential fashion to determine initiation/promotion relationships.

An intriguing feature of cancer is the occurrence of an extended period of time between the initial application or exposure of a carcinogen and the appearance of a malignant tumor.

In certain instances, the latency period between exposure and development of a tumor may be over 20 years. The two-stage theory of cancer formation was development several years ago to help explain the latency phenomenon. The two stages are divided into initiation, caused by applying a carcinogen directly to a cell population, and promotion, which occurs subsequently and results principally from the application of a co-carcinogen. Evidence supporting the concept that cancer is a multicausal, multistep process is powerful.

The primary objective of the study is to determine the ultrastructural and selected cytochemical effects of an initial acute exposure of medaka to diethylnitrosamine (48 hours at 200mg/L) followed by a chronic exposure of the fish to trichloroethylene (10mg/L). The experiment is also designed to determine whether diethylnitrosamine can serve as an inducer for the process of carcinogenesis. Trichloroethylene was chosen as a compound of interest primarily because the chemical is considered a significant contaminant of various toxic sites and also represents a serious risk to aquifers. Relatively little is known of the cytochemical and ultrastructural effects of exposure of fish model systems to trichloroethylene. The acquisition of such basic information may prove valuable to scientists studying chemically induced carcinogenesis and investigators attempting to predict the consequences of exposure to contaminants of toxic waste sites containing trichloroethylene.

Three specific enzyme systems, acid phosphatase, peroxidase and magnesium dependent, adenosine triphosphatase are being investigated ultrastructurally by means of electron-dense visual

markers. Pre-neoplastic lesions and hepatocellular carcinomas which develop subsequent to the administration of diethylnitrosamine have demonstrated altered activity of certain enzymes, including adeosine triphosphatase (Hinton et al., 1988) and acid phosphatase (Fischer et al., 1983).

BODY

The basic stock of Japanese medaka employed in this study has been maintained at the U.S. Army Biomedical Research and Development Laboratory at Fort Detrick, Maryland. Sixteen day old medaka were divided into six major groups of approximately sixty fish each. One group of fish was exposed to diethylnitrosamine (10 mg/L) for 48 hours, rinsed and maintained in a tank of fresh water. Another group has been exposed continuously to trichloroethylene (10 mg/L). A third group was exposed to diethylnitrosamine (10 mg/L) for 48 hours, rinsed in fresh water and has since been exposed continuously to trichloroethylene (10 mg/L). The remaining groups were placed in fresh water and served as controls for the various exposed groups.

At eight and sixteen weeks subsequent to the initial exposure to trichloroethylene, fifteen fish from each group were shipped to Southeastern Louisiana University. The fish were anesthetized with tricaine methanesulfonate and the livers and kidneys excised. The tissues have been processed according to the following protocols.

TRANSMISSION ELECTRON MICROSCOPY

Tissues to be analyzed by conventional transmission electron microscopy was sectioned from each organ of interest, minced and placed in a cold solution of 0.1M sodium cacodylate (pH 7.4) buffered 4% glutaraldehyde. After 2 hours of fixation, the tissues were rinsed several times in fresh buffer and post-fixed in cacodylate buffered 2% osmium tetroxide for 1 hour. The tissues were rinsed in buffer, dehydrated in a graded series of ethanol and embedded in POLY/BED 812. Thin-sections have been cut by an ultramicrotome and stained with lead citrate and uranyl acetate. Tissues have been examined and photographed with a JEOL 100S transmission electron microscope at 80kV.

SCANNING ELECTRON MICROSCOPY

Kidney and liver samples selected for analysis by scanning electron microscopy was fixed for 2 hours in a 0.1M sodium cacodylate buffered (pH 7.4) solution of 4% glutaraldehyde. Subsequent to several rinses in fresh buffer, the tissues were post-fixed for 2 hours in cacodylate buffered 2% osmium tetroxide. Tissues were rinsed and stored in buffer. Eventually the samples will be dehydrated in a graded series of ethanol, critical-point-dried and coated metallically by a sputtering unit. Samples will be examined and photographed by an AMRAY 1200B scanning electron microscope at 20kV.

CYTOCHEMISTRY

Tissues selected for cytochemical analysis were cut into 50

micrometer thick sections by means of a vibratome. Controls for all cytochemical evaluations were represented by tissues placed in an incubation medium which lacked the appropriate substrate.

Peroxidase: Portions of liver were fixed for 1 hour in a cold 0.1M phosphate buffered (pH 7.2) solution of 2% glutaraldehyde. After several washes in fresh buffer, sections of tissue were cut by a vibratome and incubated for 1 hour at 37 degrees C in a solution comprised of 0.1M phosphate buffer (pH 7.2), 5mg of 3,3'-diaminobenzidine and 1% hydrogen peroxide. The sections were washed in buffer and post-fixed for 1 hour in phosphate buffered (pH 7.2) 2% osmium tetroxide. The specimens were rinsed in buffer, dehydrated in ethanol and embedded in POLY/BED 812.

Acid phosphatase: Sections of liver were fixed for 1 hour in a cold 0.06M sodium cacodylate buffered (pH 7.2) solution of 2.5% glutaraldehyde. The specimens were rinsed several times in buffer and subsequently cut into sections by a vibratome. The tissues were incubated for 1 hour at 37 degrees C in a solution comprised of 1.25% sodium glycerophosphate, 2% magnesium chloride, 0.2M Tris/maleate buffer (pH 9.0), 1% lead nitrate and distilled water. After several rinses in buffer, the tissues were post-fixed for 45 minutes in a cacodylate buffered (pH 7.0) solution of 1% osmium tetroxide. The tissues were rinsed, dehydrated and embedded in POLY/BED 812. Thin-sections will be cut by an ultramicrotome and examined unstained.

Adenosine triphosphatase: Portions of liver and kidney were fixed for 1 hour in a cold 0.06M sodium cacodylate buffered (pH 7.0) solution of 2.5% glutaraldehyde. The fixed tissues were

rinsed several times with fresh buffer and cut into sections by a vibratome. The specimens were incubated for 1 hour at 37 degrees C in a solution consisting of 3mM ATP, 3mM magnesium sulfate, 3mM lead nitrate and 0.2M Tris/maleate (pH 7.4). The tissues were rinsed thoroughly in buffer and post-fixed for 1 hour in cacodylate buffered (pH 7.4) solution of 1% osmium tetroxide. The tissues will be rinsed, dehydrated and embedded in POLY/BED 812.

A significant factor which the Principal Investigator wishes to mention is the extended delay in initiating this project. All aspects of chemical exposures and subsequent rearing of fish were the responsibility of personnel at the Biomedical Research & Development Laboratory, Fort Detrick, Maryland. Several difficulties in rearing the fish were encountered during the early phases of the study. Fresh fish had to be obtained from the Gulf Coast Research Laboratory and, as a result, exposure to the compounds was delayed for several months. As a result, only two of the selected time points for sacrifice have reached. The Principal Investigator has devoted much of his time to processing tissue for analysis by electron microscopy. A significant number of blocks of tissue have been trimmed and are ready for thin-sectioning.

The two groups of experimental fish that have been exposed to trichloroethylene for 6 months, which represents one-half of the projected period of exposure, have exhibited no observable evidence of stress. Due to delays in initiating the study, the only ultrastructural information acquired thus far relates to control tissue which has been observed with the transmission

electron microscope. Fixation of tissue has been adequate, with no evidence of fixative related artifacts.

The liver of control medaka was comprised of hepatocytes established in a tubulosinusoidal pattern. Plates 2 cells thick extended out from a central vein. In this tubulosinusoidal configuration, the lumen of the tubule was the bile canaliculus. The space of Disse, attenuated endothelial cells and the sinusoidal lumen were positioned at the basal region of hepatocytes. Bile preductular cells formed significant segments of intralobular biliar, ductules.

Large, centric nuclei of hepatocytes contained sparse quantities of heterochromatin which, when present tended to associate with the nuclear envelope. Nucleoli were distinguished by clusters of granular elements separated by anastomosing nucleolonemas. A substantial portion of the nucleolonemas were arranged as a compact electron-dense nucleolar core. Lipid droplets were dispersed apparently at random. Lysosomes and peroxisomes were prevalent among all hepatocytes observed.

Elongated and cylindrical mitochondria were characterized by relatively short, narrow cristae and an electron-opaque matrix. Mitochondria did not demonstrate a discernible pattern of distribution. The rough endoplasmic reticulum was arranged as flattened cisternae of variable length with no detectable pattern of organization.

Bile preductular cells were long and narrow in appearance and displayed oval nuclei many of which were indented. Substantial amounts of both euchromatin and heterochromatin were dispersed in the nucleus. Tonofilaments were extensive in number

and expanded in various planes. Desmosomes were a frequent feature between adjacent hepatocytes and bile preductular cells. Tissue prepared for cytochemical analysis is currently being sectioned and analyzed.

CONCLUSIONS

Very few significant statements can be made at this point in the investigation. Initiation of the project was delayed approximately 6 months because of difficulties involving the rearing of experimental fish. Although 2 of the 4 projected sacrifices have occurred, much of the effort devoted to the project by the Principal Investigator has concentrated on processing the tissues for eventual analysis by electron microscopy.

Initial observations indicate the structural integrity of tissues has been maintained, thus, there is no need to alter concentrations or osmolarities of the fixative solutions. All control hepatic tissues appeared to be similar, ultrastructurally to liver of medaka which has been described previously. The Principal Investigator is in the process of analyzing tissue for the detection of acid phosphatase and peroxidase. According to investigators at the Biomedical Research & Development Laboratory, all fish exposed continually to trichloroethylene have displayed discernible evidence of stress or pathology. Accordingly, the experiment can proceed as originally planned.

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